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| <p>(54) Title: MODIFICATION OF POLYSACCHARIDES</p> <p>(57) Abstract</p> <p>The present invention relates to a method for manufacturing modified polysaccharides comprising of placing the polysaccharide in contact with a sugar group-transferring enzyme and a sugar group donor. The placing of the polysaccharide in contact with a sugar group-transferring enzyme and a sugar group donor can be effected <i>in vivo</i> as well as <i>in vitro</i>. The result of the method is modified polysaccharides which can be used for different food and non-food applications.</p> | | |

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MODIFICATION OF POLYSACCHARIDES

The present invention relates to the modification of polysaccharides.

Polysaccharides, such as starch, cellulose, pectin, hemicellulose and the like have a diversity of applications. Starch for instance has long formed part of the diet of humans and many animals. In addition to food applications, different non-food applications have been developed for starch and also for many other polysaccharides. The industrial use of starch in particular has increased enormously in recent years. Cellulose in turn is for instance used in the production of paper, in textiles, building materials etc.

The polysaccharides occurring in nature have specific properties which are sometimes less suitable for particular applications. In order to provide a polysaccharide with the desired properties for a particular application use is often made of chemical modification. Chemical modification has the drawback that it involves one or more additional processing steps. In addition, the chemical processing of products used in foods has a somewhat undesirable ring to the consumer nowadays.

It is the object of the invention to provide an alternative, natural manner of modifying polysaccharides. In addition, the invention has for its object to provide new polysaccharides for new application possibilities.

This is achieved by the invention with a method comprising of placing the polysaccharide in contact with a sugar group-transferring enzyme and a sugar group donor. In this manner an enzymatic modification takes place, wherein no (undesirable) chemical agents are used.

According to the invention a distinction can be made between direct and indirect modification. Direct modification entails the sugar group, which is transferred from the sugar group donor, being (covalently) linked to the polysaccharide to be modified. Indirect modification means there is no direct linking but that changes are made in the physiological appearance of the polysaccharide by the sugar group-transferring

enzyme. As example hereof can be mentioned the inclusion in a starch granule of a second polysaccharide formed by the sugar group-transferring enzyme. In addition, the transfer of a sugar group to a water molecule can also be
5 seen as an indirect modification. In such a transfer to water, sucrose is cleaved into glucose and fructose. Due to such a cleaving the physiological conditions in the plant cell change which can result in a change in the physiological appearance of the polysaccharide, such as
10 for instance in the form and size of a starch granule.

The sugar group-transferring enzyme can be any enzyme which, as the name already indicates, transfers a sugar group from a sugar group donor to an acceptor molecule. Such enzymes are usually referred to as trans-
15 ferases. The acceptor molecule will usually be the polysaccharide to be modified. In the case of indirect modification there is another acceptor, such as sucrose or glucose. In the case of transfer to water, water is the acceptor and the enzyme is usually referred to as invertase. In principle, all transferases have to a greater or
20 lesser extent the capacity to use water as acceptor. These enzymes are called transferases or invertases in accordance with the proportion of transferase or invertase activity. The sugar group for transferring can
25 consist of one or more sugar units. In the case of invertases or invertase activity only one sugar unit is transferred.

The invention relates particularly to the use of fructosyl transferases or glucosyl transferases as
30 sugar group-transferring enzymes and fructosyl donors and glucosyl donors as sugar group donor. In the continuation of the application fructosyl transferases and/or glucosyl transferases in particular will be discussed by way of example. Other sugar group-transferring enzymes can
35 however be applied instead of these without departing from the invention. The transfer of a sugar group to water is also included hereinbelow.

Fructosyl transferases (FTFs) are enzymes, which are capable of transferring fructosyl units from a fructosyl donor, for instance sucrose, to an acceptor. Fructans can inter alia be formed in this manner which consist almost wholly of repeating fructose units. The number of units in a fructan molecule can vary from 3 to more than 100,000. The type of mutual binding between the fructose units can be β -2,1 as well as β -2,6 (Suzuki & Chatterton (eds) Science and Technology of fructans, CRC Press Inc., Florida, USA (1993)).

Known fructosyl transferases are for instance levan sucrase, invertase, SST, SFT, GFT (also known now as 6G-FFT), FFT (Steinmetz et al., Mol. Gen. Genet. 200, 220-228 (1985), Angenent et al., pp. 173-184. Inulin and inulin-containing crops, A. Fuchs (Ed), Koops et al., J. Exp. Botany 45, 1623-1631 (1994), Sprenger et al., Proc. Natl. Acad. Sci. USA 92, 11652-11656 (1995)), Koops et al., Plant Physiol. 110, 1167-1175 (1996), Lüscher et al., FEBS Letters 385, 39-42 (1996), Taussig & Carlson, Nucl. Acids Res. 11, 1943-1953 (1983), Arai et al., Plant Cell Physiol. 33, 245-252 (1992), Klann et al., Plant Physiol. 99, 351-353 (1992), Zhou et al., Plant Physiol. 106, 397-398 (1994), Schwebel-Dugué et al., Plant Physiol. 104, 809-810 (1994)).

Glucosyl transferases (GTFs) can likewise use sucrose as sugar group donor (of glucosyl groups). Glucosyl transferases can result inter alia in the formation of dextrans, glucose polymers of a minimum of 2 glucose units, which can be connected α -1,6 or α -1,3. Dextrans have various applications and are used inter alia as bulk agent in plasma-replacing preparations.

Known glucosyl transferases are for instance alternan sucrase, GTF-I, GTF-S and GTF-SI (Cote, Carbo. Polym., 19, 249-252 (1992); Griffard et al., J. Gen. Micro. 139, 1511-1522 (1993)). Different enzymes result in polymers of different length and type of binding.

The fructosyl and glucosyl transferases used according to the invention can be of natural origin, i.e. plant Physiol.

isolated from a plant or micro-organism. In addition, they can be recombinantly manufactured and optionally genetically modified versions of the natural enzyme. A modified version can for instance be a fructosyl or glucosyl transferase with an increased substrate affinity. Such modified versions can for instance be produced by using gene constructs manufactured with the aid of recombinant DNA techniques and serve for instance to increase the quantity of fructose covalently linked to the polysaccharide.

Placing of the polysaccharide in contact with the enzyme can be effected in vivo as well as in vitro. The starting point in vivo is a transgenic plant cell in which the enzyme is expressed. The modified polysaccharide is produced in the plant and can easily be isolated therefrom, possibly in the same manner as the native polysaccharide would also be isolated.

An in vivo method according to the invention comprises the steps of:

- a) manufacturing a gene construct comprising at least one DNA sequence which codes for a sugar group-transferring enzyme, optionally operably linked to a targeting signal sequence, which DNA sequence is further optionally operably linked to transcription initiation signals located upstream and optionally operably linked to transcription termination signals located downstream;
- b) transforming a plant cell with the gene construct;
- c) multiplying the transformed plant cell; and
- d) optionally isolating modified polysaccharides from the plant cell.

If the DNA sequence which codes for a sugar group-transferring enzyme integrates into the plant genome, transcription initiation signals and/or transcription termination signals are not per se necessary. In such a case signals already present in the genome can be used.

Multiplying of the transformed plant cell can take place in different ways. Regeneration to plant tissue such as callus or the like or to a complete plant can thus take place. In addition, plant cells in suspension can also be multiplied. The oligosaccharide is then isolated from the tissue, the plant or the cells in suspension.

The gene construct can further also comprise at least one DNA sequence which codes for a substrate affinity-providing peptide sequence. The enzyme is hereby brought into close contact with the substrate to be modified. This enhances the modification efficiency. The substrate affinity-providing peptide sequence is operably linked to and preferably lies in reading frame with the sequence which codes for the enzyme.

In the case of starch modification the substrate affinity-providing sequence is preferably chosen from the group consisting of the maltose-binding protein of Escherichia coli, the granule-bound starch synthase protein, the starch synthase protein, the branching enzyme, the D-enzyme from plants or micro-organisms and/or fragments and/or isoforms thereof (Baba et al., Plant Phys. 103, 565-573 (1993); Boyer & Preiss, Biochem. Biophys. Res. Comm. 80, 169-175 (1978); Denyer et al., Planta 196, 256-265 (1995); Dry et al., Plant J. 2, 193-202 (1992); Kakefuda & Duke, Plant Phys. 91, 136-143 (1989)).

In the case of cellulose modification it is possible for instance to opt for the cellulose-binding domain of the glucoamylase of Aspergillus, the scaffoldine of Clostridium thermocellum (Tibtech 13, 447-450 (1995)), or of cellulases of fungi (Reinikainen et al., Proteins 14, 475-482 (1992)). These proteins or protein fragments can be linked C-terminally as well as N-terminally to the fructosyl or glucosyl transferase enzyme.

Pectin-binding domains can come from pectinases, while fructanases have fructan-binding

domains. Fructan-binding domains are for instance important when glucosyl transferases are used for modifying fructans.

By means of the targeting signal sequence the enzyme is targeted to the location of the substrate in the plant. Such targeting signal sequences can for instance be plastid-specific, cell wall-specific or vacuole-specific. Plastid-specific targeting signals are used in particular for starch modification since starch is situated in chloroplasts and amyloplasts. In contrast, cellulose is a cell wall constituent and modification thereof will take place by directing the enzyme to the cell wall. The same applies for pectin, hemicellulose, mannans, arabinans and xylans. Present in the vacuole are inter alia stachyose, verbascose, ajugose and other members of the raffinose oligosaccharide family (Bachmann & Keller, Plant Phys. 109, 991-998 (1995)). To enable enzymatic modification thereof the enzyme must be directed to the vacuole. When no targeting sequence is present in the gene construct the enzyme is expressed in the cytoplasm.

A plastid-specific targeting signal sequence preferably codes for a peptide from the group consisting of the ferredoxin of Silene pratensis, plastocyanin of Silene pratensis, ferredoxin of Arabidopsis thaliana, plastocyanin of Arabidopsis thaliana (Smeekens et al., Nucl. Acids Res. 13, 3179-3194 (1985); Smeekens et al., Nature 317, 456-458 (1985); Vorst et al., Plant Mol. Biol. 14, 491-499 (1990) and Vorst et al., Gene 65, 59-69 (1988)).

An example of cell wall-specific targeting signals is the apoplastic targeting signal of the pathogenesis-related protein S (PR-S) (Cornelissen et al., Nature 321, 531-532 (1986), and as vacuole-specific signals can for instance be mentioned the targeting signals of the proteins patatin (Sonnewald et al., Planta 179, 171-180 (1989)), sporamin (Matsuoka et al., J. Biol. Chem. 265, 19750-19757 (1990)), chitinase (Neuhaus et

al., Proc. Natl. Acad. Sci. USA 88, 10362-10366 (1991))
and lectin (Wilkins et al., Plant Cell 2, 301-313
(1990)).

For expression of the glucosyl or fructosyl
5 transferase (fusion) gene in plant tissue specific
expression signals are of course required which are
active in plants. These signals can be supplied together
with the gene construct but, if the construct integrates
into the genome, expression signals already present in
10 the genome can also be used. A well-known generally used
promoter for gene expression in plants, which can be
included for instance in the gene construct, is the 35S
promoter of the cauliflower mosaic virus. This promoter
is active in many tissues of the plant, irrespective of
15 the stage of development. In addition, strong or weak,
tissue-specific or development-regulated or other types
of promoters can be used as desired, depending on the
plant species and the purpose. In the case of
modification of starch by fructosyl or glucosyl
20 transferases in the tubers of cultivated crops such as
potato, a strong tuber-specific promoter is recommended,
such as the promoter of the granule-bound starch synthase
(Visser et al., Plant Mol. Biol. 17, 691-699 (1991)), the
patatin promoter (Sonnewald et al., Planta 179, 171-180
25 (1989)) or the sporamin promoter of Ipomoea batatas
(Nakamura et al., Plant Phys. 96, 902-909 (1991)).

In order to enable a further increase in the
transcription levels the promoter sequence is often
provided with an enhancer duplication.

30 In the case of starch modification in the seeds
of starch-containing crops, such as corn, wheat, pea or
rice, a strong seed-specific promoter is recommended
which is active in storage tissue, such as the endosperm
of corn, wheat and rice and the seed lobes of peas.

35 Examples of such promoters are for instance the seed-
specific rita-1 promoter of rice (Izawa et al., Plant
cell 6, 1277-1287 (1994)), the endosperm-specific Opaque-
2 and zein promoter from corn (Gallusci et al., Mol. Gen.

Genetics 244, 391-400 (1994), Kirihara et al., Mol. Gen. Genetics 211, 477-484 (1988), Kirihara et al., Gene 71, 359-370 (1988)). Further the β -phaseolin promoter (Riggs, Plant Sci. 63, 47-57 (1989), Buston et al., EMBO J. 10, 1469-1479 (1991)), the napin promoter (Radke et al., Theor. Appl. Genet. 75, 685-694 (1988), Kohno-Murase et al., Plant Mol. Biol. 26, 1115-1124 (1994)), the β -conglutinin promoter (Lessard et al., Plant Mol. Biol. 16, 379-413 (1991)), the lectin promoter (Okamura et al., Proc. Natl. Acad. Sci. USA 83, 8240-8244 (1986)), the phytohaemagglutinin promoter (Riggs et al., Plant Cell 1, 609-621 (1989)) and the canavalin promoter (Yamamoto et al., Plant Mol. Biol. 27, 729-741 (1995)).

The translation of the mRNAs can be improved by further adding to the construct a translational enhancer, for instance the Alfalfa Mosaic Virus RNA4 translational enhancer signal which must be present in the 5' transcribed but non-translated region.

For a correct termination of the RNA synthesis a termination signal is preferably added to the construct. An example of a suitable termination signal is the nopaline synthase gene termination sequence of Agrobacterium tumefaciens (Bevan, Nucl. Acids Res. 22, 8711-8921 (1984)).

In the in vivo method it is advantageous when the modified polysaccharides accumulate in harvestable organs, such as roots, leaves, stems, tubers, bulbs and seeds.

It is particularly surprising that it is possible according to the invention to produce fructans in plastids since it has been assumed heretofore that no sucrose or sucrose insufficient for this purpose would be present in plastids such as chloroplasts or amyloplasts. (Bird, I.F. et al., Phytochemistry 13, 59-64 (1974); Mares, D.J. et al., J. Exp. Bot. 29, 829-835 (1978)). In addition, in "Chloroplast metabolism, the structure and function of chloroplasts in green leaf cells" (Ed. Barry Halliwell, Clarendon Press, Oxford (1984) and in

"Photosynthesis, molecular, physiological and environmental processes (2nd edition) (Ed. D.W. Lawlor), Longman Scientific & Technical, Essex, UK (1993) it is demonstrated that sucrose does not diffuse through the chloroplast membrane. The skilled person would therefore expect that fructan production in plastids is not possible. Sucrose is in fact the most important fructosyl donor in vivo (Dedonder, Meth. Enzyme 8, 500-505 (1966), Edelman & Jefford, New Phytol. 67, 517-531 (1968)). Sucrose can, in the plastids, also serve as substrate for a glucosyl transferase.

It has further been found according to the invention that, in addition to the presence of an enzyme not present in the wildtype, the (growing) conditions can be of influence on the degree of modification of the polysaccharide. Genetically identical transgenic potato lines were cultivated in the summer and the winter, respectively. Of these, those cultivated in the winter were found to contain a higher fructan content than those cultivated in the summer. Analysis of the starch granules from the potatoes further showed that in the case of the winter potatoes the starch granules were comparatively smaller and moreover that the size distribution was much more uniform compared to both the wildtype and the summer potatoes. In addition, clear differences could be observed between starch from the transgenic plants and starch from wildtype plants grown under the same conditions. In this manner it becomes possible to influence the polysaccharide modification. The conditions will herein be chosen such that the desired type of influence eventually results. These desirable conditions will sometimes be sub-optimal (winter) and sometimes more optimal (summer).

The extent and nature of the modification in transgenic plants by varying the growing conditions can therefore result in inter alia smaller granules with a changed morphology. The morphology of suspended granular starch has a great influence on the rheological

properties, including the viscosity of the gel during gelatinisation (so-called top viscosity). In addition, very small starch granules or particles with a size in the order of 1 micron are stable in aqueous solutions, which gives a non-precipitating suspension (latex). This is advantageous in the final gelatinisation step, but also in the pumping and storage of starch suspensions in industrial applications. As a result of the relatively large surface/volume ratio specific applications such as biologically degradable slow-release matrix can also be developed. Considerable interest in these so-called small starch particles can be discerned in the literature in recent years. Relatively small starch granules are occasionally found in nature in exotic plants. However, this type of small starch granules is difficult to extract and moreover does not have the desired properties which potato starch possesses.

The invention therefore further relates to a population of modified starch granules which have a smaller diameter and more uniform granule size distribution compared to starch granules originating from a wildtype plant and which can be used in foodstuffs as fat substitute and to encapsulate aromatic substances and/or flavourings. They are also suitable for non-food applications such as for instance paper coatings, as separating material or column material for chromatography, as carrier material for catalysts, as carrier material for pigment, as basis for cosmetic creams, in paints, as slow release systems for agrochemicals and the like.

In gelled starch, i.e. starch heated above the gelatinisation temperature in excess water ($> 70\%$ (m/m)), starch pastes and other water-containing starch products such as bread and biscuits, retrogradation occurs after a few hours or days, this manifesting itself in the material becoming stiffer or brittle ("ageing"). The whole process of retrogradation includes the re-forming of molecular structures in (partially)

destructured systems, resulting in a change, usually seen as a deterioration, in the physical properties of the product. Examples of undesired changes are gels becoming unstable through crystallization, followed by separating
5 out of water (syneresis), bread and biscuits becoming "stale" and biologically degradable plastics on a basis of starch becoming brittle. It is assumed in the literature that retrogradation can be inhibited or prevented either by substitution of the crystallizable
10 chains in both amylose or amylopectin or by developing starches with fewer crystallizable components (amylose-free or "waxy" starches). The obtaining of derivatized or fructosylated starch, including fructosylated amylose and/or amylopectin would hereby seem very relevant for
15 both the food and the non-food sectors.

By direct or indirect modification and/or by varying the conditions in which a transgenic plant grows the retrogradation behaviour of a polysaccharide such as starch can be changed according to the invention.

20 The invention thus further relates to the use of modified polysaccharides of which the retrogradation behaviour has been changed for the improvement of the shelf-life of bakery products such as biscuits and bread, or of sauces. Polysaccharides modified in this manner can
25 further be used in non-food applications such as bioplastics, drilling liquids and glues.

In addition to the in vivo method according to the invention, the placing of the polysaccharide in
30 contact with a sugar group-transferring enzyme, such as a fructosyl transferase or glucosyl transferase, can also be effected in vitro. In practice this entails an incubation of the substrate with the enzyme under suitable conditions in respect of temperature, pH, ionic strength and the like.

35 Before using these enzymes in vitro they are preferably first fully or partly purified. Natural or heterologous hosts can be used as source. The purification of fructosyl transferases from their natural

host is for instance described for levan sucrase of B. subtilis by Ebskamp et al., Bio/techn. 12, 272-274 (1994). Other bacterial levan sucrases are for instance found in Bacillus amyloliquefaciens (Tang et al., Gene 5 96, 89-93 (1990)), Streptococcus mutans (Shiroza & Kurazitzu, J. Bacteriol. 170, 810-816 (1988)) and Zymonas mobilis.

The purification of sucrose sucrose fructosyl transferase (SST) from the onion is found in Angenent et al. on pages 173-184 of "Inulin and Inulin-containing crops", A.Fuchs (Ed.) Elsevier Science Publishers B.V. 10 (1993) or in PCT/NL95/00241. Other fructosyl transferases from plants are for instance fructan fructan fructosyl transferase (FFT), sucrose fructan fructosyl transferase 15 (SFT) and glucose fructan fructosyl transferase (GFT), which can for instance be isolated from onion, barley, wheat or Jerusalem artichoke.

When use is made of a heterologous host as source for the sugar group-transferring enzyme, this host 20 will first have to be activated to production of the desired enzyme by transformation with a construct coding for the enzyme. The enzyme can subsequently be isolated from the host in per se known ways. An example of a suitable expression system for E.coli is the QIAexpress 25 expression system (Qiagen Inc., USA).

In vitro modification of polysaccharides with a sugar group-transferring enzyme, whether or not purified, such as fructosyl or glucosyl transferase, can be performed in a manner known to the skilled person in a 30 suitable buffer, containing in any case a fructose donor. The levan sucrase of B.subtilis functions well for instance in 50 mM phosphate buffer (pH 6.0) with 1% Triton, 100 μ M PMSF and 20 mM sucrose. Fructosyl transferases can in determined conditions also transfer fructose to water 35 in addition to the polysaccharide. Because this does not enhance the efficiency of the reaction, the reaction can also be performed in a low-water or water-free environment.

In both the in vivo and in vitro method natural as well as modified versions of the sugar group-transferring enzyme can be applied, while combinations of a plurality of enzymes of eukaryotic as well as
5 prokaryotic origin can also be applied. By means of - genetic modification the properties such as substrate - affinity, activity and the like can also be influenced.

The transfructosylation or transglucosylation reaction can in principle be performed on any fructose or
10 glucose acceptor, respectively. Recommended however are polysaccharides such as starch, cellulose, fructan, pectin, hemicellulose, amylose, amylopectin etc. Starch is usually built up of two types of glucane polymers, amylose and amylopectin. Amylose consists of substantial-
15 ly unbranched chains of $\alpha 1,4$ -linked glucose residues, while amylopectin, in addition to $\alpha 1,4$ -bonds, also has $\alpha 1,6$ -bonds which link chains mutually. In addition to starch, amylose and amylopectin can also be used separately as substrate for the modification.

20 The present invention will be further elucidated by means of the following examples, which are only given by way of illustration but are not intended to limit the invention in any way whatsoever.

25 EXAMPLES

EXAMPLE 1

In vitro transfructosylation of amylose, cellulose and starch

1. In vitro transfructosylation of amylose

30 It was investigated whether the levan sucrase enzyme is capable of using amylose as fructosyl acceptor. Chosen for this experiment was the levan sucrase of Bacillus subtilis coded by the sacB gene (Steinmetz M. et al. Mol. Gen. Genet., 200:220-228 (1985)). This enzyme
35 preferably uses sucrose and fructan as fructosyl acceptor but it can use many other compounds as fructosyl acceptor in the transfructosylation reaction (Dedonder R. Meth. Enzyme. 8,500-505 (1966)).

The enzyme was purified according to the method of Ebskamp et al. (Bio/techn. 12,272-274 (1994)) and incubated with 50 mM phosphate buffer (pH 6.0), 1% triton, 100 μ M PMSF, 14 C sucrose (Amersham Int. plc, UK) and 50% acetone in the presence of 20 μ l amylose resin (New England Biolabs inc.). During the incubation for 18 hours at 30°C, 14 C labelled fructose units will be incorporated in fructan and covalently linked to amylose. So as to be able to distinguish between 14 C labelled fructan which is covalently linked to the amylose resin and 14 C fructan which is captured in the amylose resin, purified levan sucrase of Bacillus subtilis was likewise incubated with 50 mM phosphate buffer (pH 6.0), 1% triton, 100 μ M PMSF, 14 C sucrose and 50% acetone in the absence of 20 μ l amylose resin. After incubation for 18 hours at 30°C the amylose resin was added to the reaction mixture. Both tubes with amylose resin were then washed repeatedly with 100 volumes of water. Since bacterial fructan is water-soluble, non-covalently linked 14 C-labelled fructose and fructan is therefore largely washed away while all the covalently linked 14 C-labelled fructose and fructan will remain behind in the amylose resin pellet.

Determination of the activity of the amylose resin by scintillation count revealed that the activity of the resin which was incubated in the presence of fructosyl transferase was 35 times higher than the activity of the amylose resin which was added after the 18 hour incubation period at 30°C of the levan sucrase enzyme (table 1). This demonstrates that the levan sucrase enzyme of Bacillus subtilis is indeed capable of using amylose as fructosyl acceptor in the transfructosylation reaction.

Table 1

Incubation with fructosyl transferase

| | |
|-----------------|---|
| | nmol fructose bound to amylose resin |
| with resin | 20 |
| 5 without resin | 0.6 |

2. In vitro transfructosylation of starch and cellulose

In similar manner it was investigated whether the levan sucrase enzyme is also capable of using insoluble corn starch or insoluble cellulose isolated from cotton as fructosyl acceptor in vitro.

The experiment was performed in the same manner as the experiment with amylose, with the difference that, instead of with amylose, the enzyme was incubated with either 10 mg insoluble corn starch (Sigma Chem. Co, St. Louis, MO63178, USA) or 10 mg insoluble cellulose isolated from cotton (Bio-Rad, Richmond, CA, USA).

Determination of the activity of the starch or the cellulose by scintillation count revealed that the activity of the material incubated in the presence of fructosyl transferase was respectively 8 and 19 times higher than the activity of the material added after the 18 hour incubation period at 30°C of the levan sucrase enzyme (table 2), while TLC analysis revealed that the total fructan synthesis was constant in all reaction mixtures. This demonstrates that the levan sucrase enzyme of Bacillus subtilis is indeed capable of using starch and cellulose as fructosyl acceptor in the transfructosylation reaction.

Table 2

Incubation with starch and cellulose

5

| Incubation with fructosyl transferase | nmol fructose per mg fructosyl acceptor |
|---------------------------------------|---|
| with cellulose | 57 |
| without cellulose | 3 |
| with starch | 70 |
| without starch | 9 |

10

EXAMPLE 2In vitro transglucosylation of amylose

15

In the same manner as described in example 1, under 1., it was investigated whether the dextran sucrase of Leuconostoc mesenteroides (Kobayashi et al., Biophys. Acta 614, 46 (1980)) can transfer glucose of the glucose-donor sucrose to the glucose-acceptor amylose. Dextrans should then be formed.

20

The enzyme came from Sigma-Aldrich N.V./S.A. and was tested in reaction mixtures of 50 μ l. Each reaction mixture contains 150 mM 14 C sucrose (Amersham Int. plc, UK), 15 mM NaAc (pH 5.2), 0.5 units of dextran sucrase, with or without 20 μ l amylose resin. The whole was incubated overnight at 30°C and treated further as stated in example 1.

25

Table 3 shows that in the presence of amylose resin 5 times as much radioactivity is incorporated. This means that the enzyme can use amylose as glucose-acceptor.

Table 3

| | nmol glucose bound to amylose resin |
|---------------|-------------------------------------|
| with resin | 13.0 |
| without resin | 2.6 |

5

EXAMPLE 3**Expression of sacB in plastids of tobacco plants****1. Selection of the gene**

Starch biosynthesis takes place in the
 10 plastids. In order to express fructosyl transferases in
 the plastids the levan sucrase enzyme was chosen which
 was coded by the sacB gene of Bacillus subtilis (Chambert
 et al. Biochimica et Biophysica Acta 1132, 145-153
 (1992)). In order to send the levan sucrase to the
 15 plastids the ferredoxin targeting signal of Silene
pratensis (Smeekeens et al., Nucl. Acid. Res. 13:3179-3194
 (1985)) was chosen and coupled in reading frame to the
 levan sucrase gene.

20 2. Construction of 35S-fd-sacB-NOS in the binary vector (pFD-LS)

Plasmid pSTU8 was constructed by cloning a 1.3
 kb EcoRI-NdeI fragment from plasmid pGMK52 (Chambert et
 al. Biochimica et Biophysica Acta 1132, 145-153 (1992)),
 25 which codes for the levan sucrase enzyme, in plasmid
 pMTL22 digested with EcoRI and NdeI (Chambers et al.,
 Gene 68, 139-149 (1988)). The 120 bp NcoI-EcoRI fragment
 of plasmid pKK3 (Ebskamp et al. Bio/Techn 12, 272-274
 (1994)) was subsequently cloned in the plasmid pSTU8
 30 digested with NcoI and EcoRI, resulting in plasmid pSTU-
 22. The 1.6 kb NcoI-BamHI fragment from pSTU22 was cloned
 in the expression vector pPA2 (Ebskamp et al., Bio/techn.
 12, 272-274 (1994)) resulting in plasmid pSTU42.

Plasmid pSTU94 was constructed by cloning the
 35 0.5 kb NcoI-BamHI fragment, which codes for the

ferredoxin peptide of Silene pratensis from pETFD100 (Pilon et al., J. Biol. Chem. 270, 3882-3893 (1995)), in vector pMTL22 digested with NcoI and BglII. In order to clone the ferredoxin transit sequence for the sacB gene the 1.9 kb NcoI-XhoI fragment of the vector pSTU42 was isolated and cloned in the vector pSTU94 digested with Eco47III and XhoI after the NcoI restriction site was blunted with Mung bean nuclease, in order to provide pSTU113. To clone the 35S promoter for the fd-sacB-NOS construct the 2.1 kb NcoI-XhoI fragment from plasmid pSTU113 was cloned in vector pPA2 digested with NcoI and XhoI in order to produce pSTU176. Subsequently The 3.0 kb SmaI-XhoI 35S-fd-sacB-NOS construct was then cloned in the binary vector pVMJ5, resulting in pFD-LS (fig. 1).

The pFD-LS plasmid was transformed by means of electroporation (Mattanovich et al. Nucleic Acids Res. 17,6447 (1989)) in Agrobacterium tumefaciens strain LBA4404. The construct was introduced into Nicotiana tabacum var. Samson NN using the leaf disc transformation method (Horsch et al. Science 227, 1229-1232 (1985)). Regenerated plants, which are referred to as FD-LS plants, were selected for kanamycin resistance and cultured on MS medium (Murashige and Skoog Physiol. Plant. 15, 473-497 (1962)).

25

3. Analysis of transgenic tobacco plants

FD-LS plants were cultured both in tissue culture and in the greenhouse. Leaf material of greenhouse plants was cut off and ground in an eppendorf tube. After centrifugation for 5 minutes at 16000 rpm 1 μ l of the supernatant was spotted on TLC (Caims A.J. and Pollock C.J. New Phytol. 109, 399-405 (1988)). The TLC was developed three times in 85:15 acetone:water and subsequently treated with atomized urea as already described by Wise C.S. et al., Analytical Chemistry 27, 33-36 (1955). This method stains mainly fructose and fructose-containing polymers. While no fructan accumulation could be detected in the wildtype strain,

the screening of the transformants with the use of this method showed an extensive accumulation of fructans in these plants in an excess of 10% of the dry weight (fig. 2).

5 In order to determine whether the fructans which are present in transgenic plants were localized in the chloroplast, chloroplasts were isolated. Protoplasts were first isolated from tissue culture plants by incubating small pieces of leaf in K3 medium (Bagy, JL
10 and Maliga, P.Z. Pflanzenphysiol. 78,453-455 (1976)) supplemented with 0.4 M sucrose, 1.2% cellulase and 0.4% macerozym (K3S). During the overnight incubation these enzymes digested the plant-cell wall. Intact protoplasts have the feature that they float on K3S medium when they
15 are centrifuged for 10 minutes at 600 rpm (80 g). Isolated protoplasts were washed with W5 medium (Menczel et al. Theor. Appl. Genet. 59, 191-195 (1981) and precipitated by centrifuging for 5 minutes at 600 rpm. The protoplasts were resuspended in 2 ml isolation buffer
20 (0.33 M Sorbitol, 50 mM Hepes/KOH pH 7.3, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 0.1% BSA) and lysated by pressing the protoplasts through a small capillary. Intact chloroplasts which were present in this lysate were isolated by separation of the organelles on a continuous
25 Percoll gradient (Bartlett, S.C., Grossman, A.R. and Chua, N-H in: Hallick, R.B. and Chua, N-H. (eds) Methods in Chl. Mol. Biol. Elsevier Biomed. Press Amst./New York/Oxford pp 1981-1091 (1982)). Intact chloroplasts isolated from the gradient were washed by centrifuging
30 them through 40% Percoll solution. A part of the chloroplast isolate was subsequently used for chlorophyll determination (Arnon DI, Plant Phys. (Bethesda) 41,1-14 (1949)). Based on quantities of chlorophyll, fructans were isolated from equal quantities of chloroplasts and
35 protoplasts by extracting them three times with water. TLC analysis (fig. 2) showed that the sample containing the isolated chloroplasts has the same quantities of fructans as intact protoplasts based on the quantity of

chlorophyll. This indicates that the ferredoxin levan sucrase enzyme is present and active in chloroplasts.

5 **EXAMPLE 4**

Expression of sacB in plastids of potato plants

1. Introduction

In the same manner as in Example 3 the same gene construct was introduced into the potato Solanum
10 tuberosum (var. kardal). Regenerated plants are referred to as FD-LS-A plants. Of one of these strains a number of plants was grown in the winter, while a number of genetically identical plants were grown in the summer. These plants are designated below as FD-LS-A-W or 192-2, re-
15 spectively FD-LS-A-Z or 192-3.

2. Fructan accumulation

FD-LS-A potato plants were cultured both in tissue culture and in the greenhouse. Leaf material of
20 greenhouse plants was cut off and treated in the same manner as the leaf of tobacco plants (see Example 3, under 3.). In wildtype potato plants no fructan accumulation can be demonstrated. In the transgenic FD-LS-A potato plants fructans accumulate in an excess of 5%
25 of the dry weight (Fig. 3).

3. Analysis of starch from transgenic potato tubers

The fructan level of plants grown in the winter was 5% of the dry weight, while that of plants grown in
30 the summer amounted to only 1%. It follows herefrom that growing conditions play an indirect part in the amount of fructan which accumulates.

In addition, starch granules were isolated by cutting the tubers of FD-LS-A-W, FD-LS-A-Z and wildtype
35 potatoes into large pieces and then carefully breaking the cells by a few short pulses at low speed with a polytron in 50 ml isolation buffer (50 mM Tris-HCl pH 7.4, 10 mM EDTA, 1 mM NaSulphite, 1 mM DTT). The homoge-

nate was filtered through two layers of Miracloth (Cal-biochem-novabiochem Corp., La Jolla, CA, 92039, USA). The whole was subsequently left to stand for 48 hours whereby the starch granules precipitated. Thereafter the supernatant of the FD-LS-A plants, in which the soluble fructans are situated, was pipetted off from the starch granules. The starch was successively washed with 50 ml isolation buffer, three times 50 ml water and 50 ml acetone and suspended in water to a concentration of 0.5 mg starch/ μ l water. After each washing step the starch granules were left to precipitate for 48 hours. Roughly 5 mg of the thus obtained starch granules was stored without further treatment for electron-microscopy, polarization-microscopy and fructan detection. The remaining part was conditioned at 58% relative air humidity and 20°C to a constant moisture content. This material was used for the other analyses.

3.1. Association of fructan to starch

Using a fructan-specific antibody it was determined whether fructans are associated to the FD-LS-A-W starch. For this purpose the structure of the starch granule was partially broken by incubating 2 μ l of the FD-LS-A-W starch for 1 hour at 75°C in 20 mM NaOH and spotting this on a nitrocellulose filter. In order to be able to distinguish between fructan which is associated to the starch granule and fructan which only adheres to the starch, a 5% fructan solution was added to wildtype potato starch. This starch was subsequently washed in the above described manner. 2 μ l of this washed starch was then also incubated for an hour at 75°C in 20 mM NaOH and spotted on the nitrocellulose filter. As positive control 2 μ l of a 5% fructan solution was spotted on the nitrocellulose filter. This filter was then incubated for 30 minutes at 120°C to bind the sugars to the nitrocellulose. Thereafter the filter was incubated for 4 hours in 5% protifar (Nutricia) to block all remaining antibody binding sites. The filter was thereafter incubated for

18 hours at room temperature with a 1/1000 dilution of the fructan-specific antibody (I_G, J606 of Organon Technica Corp., (West Chester, Penn. USA). The filter was then washed 4 times for 15 minutes in TBST solution (0.15 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Tween 20) and incubated for 2 hours with a second antibody labelled with alkaline phosphatase. The filter was subsequently washed again 4 times for 15 minutes in TBST solution. In order to detect the enzyme alkaline phosphatase the blot was stained with the reagent nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in alkaline phosphatase buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl) (Promega Corp., Madison, USA). After staining of the blot the fructan in the starch granule can be demonstrated.

After staining it can be seen on the filter that the starch isolated from transgenic FD-LS plants reacts specifically with the fructan-specific antibody while no fructan can be detected in the wildtype starch granules (fig. 4). This means that the soluble fructan does not adhere to the starch granule, but that in the starch isolated from the FD-LS plants the fructan is strongly associated to the starch granule, at least so strongly that it cannot be washed away.

Starch from FD-LS-A-W and wildtype potato plants was then incubated for one, two or three hours at 75°C in 20 mM NaOH. Longer incubation time in 20 mM NaOH at 75°C break the starch granules even further open and thus make them more accessible to the fructan-specific antibody. After the treatment with caustic soda both starch samples were baked on the nitrocellulose filter and treated as described above. It can be seen on the filter that longer incubation times amplify the fructan-specific signal (fig. 5). This means that the fructan does not lie on the surface of the starch granule but is included in the starch granule.

3.2. Shape and size of the starch granules

Using Scanning Electron Microscopy (SEM) analysis, the shape and size of the starch granules from the different sources were examined. For this purpose

5 starch from the FD-LS-A-W, FD-LS-A-Z and wildtype plants were dried in air for about 24 hours and subsequently mounted on copper sample holders using double-sided adhesive tape. Prior to the analysis the samples were sputtered with gold under vacuum. Analysis of the samples

10 shows that the wildtype starch is comparable in morphology to regular potato starch with oval-like to round smooth granules in the size of 5 to 60 micron. However, the starch from the FD-LS-A plant has a clearly differing morphology. In both samples the fraction of

15 small granules (circa 1-10 micron) has increased in quantity relative to the quantity of small granules in wildtype starch. The lower limit in the size of the granules is also lower than for the wildtype starch (respectively 1 and 5 micron). The large starch granules

20 (larger than 15 micron) from the FD-LS-A-Z plants are elongate in form with a length about three times greater than the width, while the small starch granules (1-10 micron) are round. In contrast to the other starches, the starch from the FD-LS-A-W plants has a

25 rough surface, which varies from very rough with deep cavities to slightly etched (Fig. 6).

For analysis under a polarization microscope, several droplets of the starches in water were arranged on an object slide, mixed with glycerol and covered with

30 a cover glass. The preparations were analysed on a Zeiss Axioplan microscope at magnifications in the order of 50 to 400 times with crossed polaroids as well as with phase contrast. The preparations were examined visually for the presence of double-refracting structures in the granules

35 (so-called Maltese crosses). It was found herewith that all starches consist of double-refracting granules, which indicates a spherulitic, concentrically grown structure of the granules (Fig. 7).

Of the material conditioned at 48% relative humidity and 20°C, diffractograms were recorded on a Philips PC-APD diffractometer in the symmetrical reflection mode. Diffractograms were recorded in the angular range of 4-40°C (2 θ) with a step size of 0.05°C (2 θ) and a speed of 1.5°C (2 θ)/minute. The diffractometer is equipped with a copper anode (λ_{Cu} is 0.1542 nm) and an Anton Paar TTK temperature chamber. Detection took place with a proportional detector. It was found herewith that the crystal structure and the crystallinity of the FD-LS-A-Z samples do not differ from those of the wildtype starch. Both samples have a B-type crystalline organisation, which indicates that the chain length distribution of the amylopectin in the FD-LS-A-Z starch does not differ greatly from the wildtype starch (Fig. 8A & 8B).

3.3. Amylose content

As measure of the amylose content of the starch the lysolecithin-complexing capacity (LLC) was determined. This was done by accurately weighing 6 to 7 mg starch and mixing it with a solution of 38.5 mg L- α -lysophosphatidylcholine (L- α -lysolecithin type I from egg yolk; Sigma [9008-30-4]) in 418 mg demineralized water. The sample was carefully mixed and on a Perkin Elmer DSC7 equipped with an automatic sample exchanger and calibrated at 10°C/minute with Gallium (T_{onset} 36.9°C and Indium (T_{onset} 166.7°C, ΔH 29.06 J/g)) the samples were heated from 20 to 170°C. At 170°C cooling took place to 20°C at 200°C per minute and scanned to 150°C. From the enthalpy of the endotherm at about 109°C (which indicates "melting" of complexes of L- α -lysolecithin and amylose) the LLC was determined as a measure for the amylose content after correction for the moisture content. The LLC was expressed in relation to the complexing capacity of amylose from potato starch. Table 4 shows that the LLC of FD-LS- Z-A starch is lower than that of the wildtype starch. The LLC of wildtype starch is virtually

comparable to the LLC (20-24%) observed in regular potato starch.

Table 4

| | | | |
|---|-----------|--------------------------------|------------------------------------|
| 5 | Starch | ΔH (J/g dry substance) | LLC*% (relative to potato amylose) |
| | wildtype | 5.1 (\pm 0.6) | 24 (\pm 3) |
| | FD-LS-A-Z | 3.4 (\pm 0.6) | 16 (\pm 3) |

The low LLC of the FD-LS-A-Z starch points either to a significantly lower amylose content or to a slight branching of the amylose, whereby complexing is prevented and the amylose content appears to be lower. This shows in any case that the FD-LS-A-Z starch clearly differs from the wildtype starch.

15

EXAMPLE 5

Modification of starch by FD-LS-MBP

1. Selection of the gene

Starch biosynthesis takes place in the chloroplast. In order to modify the starch by the action of fructosyl transferases the levan sucrase enzyme was chosen which is coded by the sacB gene of Bacillus subtilis (Chambert et al. Biochimica et Biophysica Acta 1132, 145-153 (1992)).

In addition to the ferredoxin targeting signal of Silene pratensis, (Smeekens et al., Nucl. Acid Res. 13:3179-3194 (1985)) to target the levan sucrase to the plastids, the affinity of the levan sucrase for the glucose polymer was increased by including in the construct the gene which codes for the maltose binding protein (malE-gene) (Duplay et al. J. Biol. Chem. 259, 10606-10613 (1984)) of E. coli.

2. Construction of 35S-fd-sacB-malE-NOS in the binary vector (pFD-LS-MBP)

35

To make the 35S-fd-sacB-malE-NOS construct a 1110 bp NruI-BamHI fragment which codes for the maltose binding protein was generated by means of PCR. The primers which were used to amplify the fragment were

1:5' GGGGGT**CGCG**AAAATCGAAG 3';
and
2:5' CCCC**GGATCC**GAATTATCAAATCC 3'.

The NruI and BamHI restriction sites (in bold print) are present in the primers 1 and 2, respectively, and were used to clone the fragment in the plasmid pSTU176 digested with EcoR5 and BamHI. The 4.1 kb XbaI-XhoI fragment was subsequently cloned in pVMJ5 digested with XbaI-XhoI to make pFD-LS-MBP (Fig. 1). This plasmid was then transformed in Agrobacterium tumefaciens strain LBA4404 and introduced into Nicotiana tabacum var. Samson NN as described in example 2. Regenerated plants were named FD-LS-MBP plants.

3. Analysis of transgenic FD-LS-MBP plants

Screening of the transgenic FD-LS-MBP plants for fructan accumulation as described in example 2 revealed that fructans can be detected in these plants.

In order to determine whether these plants likewise contain fructose in their starch granules, leaves of greenhouse plants were used for starch granule isolation. To isolate the starch granules the leaves were carefully broken by a few short pulses at low speed with a Polytron in isolation buffer (0.33 M Sorbitol, 50 mM Hepes/KOH pH 7.3, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA and 0.1% BSA). The homogenate was filtered through 2 layers of Miracloth (Calbiochem-Novabiochem Corp. La Jolla, CA, 92039 USA) and centrifuged at 4000 x g for 10 minutes. The crude extract was resuspended in a few millilitres of isolation buffer and starch granules were isolated by sedimentation in a 100% Percoll solution. The starch granules were washed with water. They were then hydrolyzed with H₂SO₄.

and the glucose and fructose content was determined by HPLC. Starch granules of wildtype tobacco plants contain no fructose but in purified starch granules of the FD-LS-MBP plants significant fructose levels could be detected.

- 5 This demonstrates that the levan sucrase enzyme is capable of transfructosylating starch in vivo or that fructan molecules are taken up in the starch granules.

EXAMPLE 6

10 Production of modified starch in transgenic crops

1. Selection of the genes and the plant species

- In order to test whether starch can also be modified in transgenic crops, such as potato, corn or wheat, Solanum tuberosum var. Karda and Zea mays were
15 selected. In order to modify starch in plants of these crops through the action of fructosyl transferases the levan sucrase enzyme was chosen which is coded by the sacB gene of Bacillus subtilis (Chambert et al., Biochimica et Biophysica Acta 1132, 145-153 (1992)). For
20 tuber-specific expression in the potato a strong tuber-specific promoter, the sporamin promoter of Ipomoea batatas, was used. For a strong expression in the endosperm of corn the zein promoter of Zea mays (Wandelt et al., Nucl. Acids Res. 17, 2354 (1989)) was chosen.

- 25 The ferredoxin targeting signal from Silene pratensis (Smeekens et al., Nucl. Acid Res. 13:3179-3194 (1985)) was once again chosen to direct the levan sucrase to the plastids. To increase the affinity of the levan sucrase for the glucose polymer the maltose binding
30 protein of E. coli coded by the malE-gene (Duplay et al., J. Biol. Chem. 259, 10606-10613 (1984)) was also used here.

2. Construction of the gene constructs

Plasmids pSP-FD-LS and pSP-FD-LS-MBP (Fig. 1) were constructed by replacing a 0.8 kb EcoRI-NcoI fragment with the 35S promoter of respectively pFD-LS and pFD-LS-MBP by a 1.0 kb EcoRI-BspHI fragment of pIMO23 (Hattori et al., Plant Mol. Biol. 5,313-320 (1985)), which contains the sporamin promoter of Ipomoea batatas. The 1.0 kb EcoRI-BspHI fragment with the sporamin promoter was generated by PCR amplification. The primers which were used for the amplification are:

1: 5' GGCTGCAGGAATTCGATATCAAGC 3'; and

2: 5' GTGAGGGCTTTCATGATGGCAGATGAGA 3'.

The restriction sites which were used to clone the fragment are designated with bold print.

Plasmids pSP-FD-LS and pSP-FD-LS-MBP were subsequently transformed in Agrobacterium tumefaciens strain LBA4404 and introduced into Solanum tuberosum var. Kardal as described (Visser, pp:1-9. Plant Tissue Culture Manual B5, K. Lindsey (Ed), Kluwer Acad. Publ. Dordrecht, Netherlands (1991)). The regenerated plants were named respectively SP-FD-LS and SP-FD-LS-MBP.

Plasmids pZE-FD-LS and pZE-FD-LS-MBP (fig. 1) were constructed by replacing a 0.8 kb EcoRI-NcoI fragment with the 35S promoter of respectively pFD-LS and pFD-LS-MBP by a 2.0 kb EcoRI-BspHI fragment containing the zein promoter of Zea mays. The 2.0 kb EcoRI-BspHI fragment with the zein promoter was generated by PCR amplification. The primers which were used for the amplification are:

1: 5' GGCTGCAGGAATTCACCTCAATCAT 3'; and

2: 5' ACCTTGCTAGTCATGATTGTTAGGTCGT 3'.

35

The restriction sites which were used to clone the fragment are designated with bold print.

Plasmids pZE-FD-LS and pZE-FD-LS-MBP were subsequently transformed in Zea mays, as described (Hill et al., Euphytica 85, 119-123 (1995)). The regenerated plants were named respectively ZE-FD-LS and ZE-FD-LS-MBP.

5

3. Starch analysis of transgenic potato and corn plants

Screening of transgenic SP-FD-LS and ZE-FD-LS plants for fructan accumulation as described in example 2 revealed that fructans can be detected in these plants.

10 In order to determine whether the SP-FD-LS-MBP and ZE-FD-LS-MBP plants likewise contain fructose in their starch granules, leaves of greenhouse plants were used for starch granule isolation as described in example 3. The starch granules were purified and hydrolyzed with H_2SO_4 and the glucose and fructose content was determined by HPLC. Although starch granules of wildtype plants never contain fructose, fructose could be detected in the starch granules of both the SP-FD-LS-MBP and the ZE-FD-LS-MBP plants. This demonstrates that also in cultivated crops, such as potato and corn, starch can be modified in vivo either by covalent linking of the fructan molecules to the starch or by inclusion of fructans in the starch granule or indirectly by changing the physiological conditions.

25

LEGEND

Fig. 1 is a schematic representation of the plasmids used.

Fig. 2 shows a TLC analysis with fructans isolated from protoplasts (T) and purified chloroplasts (C) of wild-type tobacco plants (WT) and transgenic FD-LS plants (fd-ls) plants. Fructans were isolated from equal quantities of protoplasts and chloroplasts (based on the quantity of chlorophyll). Fructans which are larger than 20 fructose units remain behind on the application site. H = fructans isolated from Helianthus tuberosus; F = fructose; and S = sucrose.

35

Fig. 3 shows a TLC analysis with fructans isolated from wildtype (WT) and transgenic FD-LS-A (fd-LS) potato plants. Fructans were isolated from equal quantities of leaf material. The fructans larger than 20 fructose units remain behind on the application site. M = fructans isolated from Helianthus tuberosus.

Fig. 4 shows the immunodetection of fructans in modified starch using a fructan-specific antibody. Starch granules were isolated from equal quantities of wildtype (C) and transgenic FD-LS-A-W (Fd-LS) potato tubers and prior to washing of the starch granules a 5% fructan solution was added to the wildtype starch. About 1 mg of the washed starch granules (G) was spotted on nitrocellulose next to 2 μ l of a 5% fructan solution (S). Fructan present on the blot was made visible with a staining reaction.

Fig. 5 shows the immunodetection of fructans in wildtype (WT) and transgenic FD-LS-A-W (fd-LS) starch granules. Isolated starch granules were incubated for 1, 2 or 3 hours in 20mM NaOH at 75°C to break open the starch granules. In order to assess whether the immunogenicity of the fructan changed under these conditions, 2 μ l of a 5% fructan solution was incubated for 1, 2 or 3 hours in 20mM NaOH at 75°C. After blotting on nitrocellulose the presence of the fructan was examined with a fructan-specific antibody. S is 2 μ l of a 5% fructan solution.

Fig. 6 shows SEM recordings of the starches wildtype, 192-3 (= FD-LS-A-Z) and 192-2 (= FD-LS-A-W) a: wild type starch (300x), b: wild type starch (600x), c: 192-3 starch (300x), d: 192-3 starch (600x), e: 192-2 starch (300x), f: 192-2 starch (600x).

Fig. 7 shows polarization microscopy recordings (20x enlarged) of wildtype starch (a), 192-3 starch (b) and 192-2 starch (c).

Fig. 8 shows diffractograms of wt starch (a) and 192-3 starch (b).

CLAIMS

1. Method for manufacturing modified polysaccharides comprising of placing the polysaccharide in contact with a sugar group-transferring enzyme and a sugar group donor.

5 2. Method as claimed in claim 1, characterized in that the placing of the polysaccharide in contact with a sugar group donor and a sugar group-transferring enzyme can be effected in vivo in a transgenic plant cell in which a sugar group-transferring enzyme is expressed.

10 3. Method as claimed in claim 2, comprising the steps of:

a) manufacturing a gene construct comprising at least one DNA sequence which codes for a sugar group-transferring enzyme, optionally operably linked to a targeting signal sequence, which DNA sequence is further optionally operably linked to transcription initiation signals located upstream and optionally operably linked to transcription termination signals located downstream;

15 b) transforming a plant cell with the gene construct;

c) multiplying the transformed plant cell; and

20 d) optionally isolating modified polysaccharides from the plant cell.

4. Method as claimed in claim 3, characterized in that the transformed plant cell is multiplied by regeneration to a plant tissue or complete plant or in cell suspension.

5. Method as claimed in claim 3 or 4, characterized in that the gene construct further comprises at least one DNA sequence operably linked to and preferably in reading frame with the gene which codes for a substrate affinity-providing peptide sequence.

6. Method as claimed in claim 5, characterized in that the substrate affinity-providing peptide sequence for the substrate starch is chosen from substrate-binding domains of proteins from the group consisting of the

maltose-binding protein of Escherichia coli, the granule-bound starch synthase protein, the starch synthase protein, the branching enzyme, the D-enzyme, which sequences can come from plants or micro-organisms and can be fragments and/or isoforms thereof.

7. Method as claimed in claim 5, characterized in that the substrate affinity-providing peptide sequence for the substrate cellulose is chosen from cellulose-binding domains of proteins from the group consisting of glucoamylase of Aspergillus, scaffoldine of Clostridium thermocellum or of cellulases of fungi, which sequences can come from plants or micro-organisms and can be fragments and/or isoforms thereof.

8. Method as claimed in claim 5, characterized in that the substrate affinity-providing peptide sequence for the substrate pectin is chosen from the substrate-binding domains of the pectinases, which sequences can come from plants or micro-organisms and can be fragments and/or isoforms thereof.

9. Method as claimed in claim 5, characterized in that the substrate affinity-providing peptide sequence for the substrate fructan is chosen from the fructan-binding domains of the fructanases, which sequences can come from plants or micro-organisms and can be fragments and/or isoforms thereof.

10. Method as claimed in any of the claims 3-9, characterized in that the targeting signal sequence is plastid-specific, cell wall-specific or vacuole-specific.

11. Method as claimed in claim 10, characterized in that the plastid-specific targeting signal sequence codes for a peptide from the group consisting of the ferredoxin of Silene pratensis, plastocyanin of Silene pratensis, ferredoxin of Arabidopsis thaliana, plastocyanin of Arabidopsis thaliana.

12. Method as claimed in claim 10, characterized in that the cell wall-specific targeting

signal sequence codes for the apoplastic targeting signal PR-S.

13. Method as claimed in claim 10,
characterized in that the vacuole-specific targeting
5 signal sequence codes for a peptide from the group
consisting of the targeting signals of the proteins
patatin, sporamin, chitinase and lectin.

14. Method as claimed in claim 1, characterized
in that the placing of the polysaccharide in contact with
10 a sugar group-transferring enzyme is effected in vitro in
the presence of a sugar group donor.

15. Method as claimed in any of the claims 1-
14, characterized in that the sugar group-transferring
enzyme is a fructosyl transferase and the sugar group
15 donor is a fructosyl donor.

16. Method as claimed in any of the claims 1-
14, characterized in that the sugar group-transferring
enzyme is a glucosyl transferase and the sugar group
donor is a glucosyl donor.

17. Method as claimed in any of the claims 1-
15, wherein the fructosyl transferase is chosen from the
group consisting of levan sucrase, sucrose sucrose fruc-
tosyl transferase (SST), fructan fructan fructosyl trans-
ferase (FFT), sucrose fructan fructosyl transferase (SFT)
25 and glucose fructan fructosyl transferase (GFT), or
modified versions thereof, from plants or micro-organ-
isms.

18. Method as claimed in any of the claims 1-14
and 16, wherein the glucosyl transferase is chosen from
30 the group consisting of alternan sucrase, glucosyl
transferase-I (GTF-I), glucosyl transferase-S (GTF-S),
glucosyl transferase-SI (GTF-SI), or modified versions
thereof, from micro-organisms.

19. Method for manufacturing modified
35 polysaccharides, in particular modified starch granules,
comprising of:

a) culturing under suitably chosen conditions a
transgenic plant, plant cell or plant tissue in the cells

of which a DNA sequence is situated which codes for a sugar group-transferring enzyme, optionally operably linked to a targeting signal sequence, which DNA sequence is further operably linked to transcription initiation signals located upstream thereof and optionally operably linked to transcription termination signals located downstream; and

b) isolating modified starch granules from the transgenic plant cell, the transgenic plant tissue or the transgenic plant.

20. Method as claimed in claim 19, wherein the suitably chosen conditions consist of conditions wherein the growth is inhibited, for instance wintery conditions.

21. Modified polysaccharide obtainable by performing the method as claimed in any of the foregoing claims.

22. Modified polysaccharide as claimed in claim 19, 20 or 21, further modified by chemical processing.

23. Use of polysaccharides as claimed in claim 21 or 22 in foodstuffs, for instance as stabilizer, thickener, gelling agent, nutritional fibre, low-calory filler or pre-bioticum.

24. Use of polysaccharides as claimed in claim 21 or 22 in non-food applications, for instance as biodegradable adhesive, flocculant, dispersant, chelator or for instance heavy metal ions.

25. Modified polysaccharides as claimed in claim 21, obtainable by using starch as starting polysaccharide in the method as claimed in claims 1-20.

26. Modified polysaccharide, particularly starch, as claimed in claim 25 having a differing retrogradation behaviour relative to the corresponding non-modified polysaccharide, particularly starch.

27. Use of modified polysaccharide as claimed in claim 26 in the improvement of the shelf-life of bakery products such as biscuits and bread, or of sauces.

28. Use of modified polysaccharide as claimed in claim 26 in non-food applications such as bioplastics, drilling liquids and glues.

29. Population of modified starch granules
5 which have a smaller diameter and more uniform granule size distribution compared to starch granules originating from a wildtype plant.

30. Population as claimed in claim 29,
obtainable by means of the method as claimed in claim 19
10 or 20.

31. Use of the population as claimed in claim 30 in foodstuffs as fat substitute and to encapsulate aromatic substances and/or flavourings.

32. Use of the population as claimed in claim
15 30 in non-food applications, for instance as paper coatings, as separating material or column material for chromatography, as carrier material for catalysts, as carrier material for pigment, as basis for cosmetic creams, in paints, as slow release systems for agrochemicals
20 and the like.

33. Transgenic plant of part thereof comprising
at least one plant cell which harbours a gene construct
comprising at least one DNA sequence which codes for a
fructosyl transferase, which DNA sequence is operably
25 linked to transcription initiation signals located upstream and optionally operably linked to transcription termination signals located downstream, and wherein the DNA sequence is optionally further operably linked to a
targeting signal sequence and optionally to a sequence
30 which is in reading frame with the DNA sequence and which increases the substrate affinity of the coded product.

34. Transgenic plant as claimed in claim 33,
wherein the product encoded by the gene construct is
expressed.

35. Reproductive components of a plant as
claimed in claim 33 or 34, chosen from the group
consisting of seeds, cuttings, tubers, bulbs, runners,
meristem.

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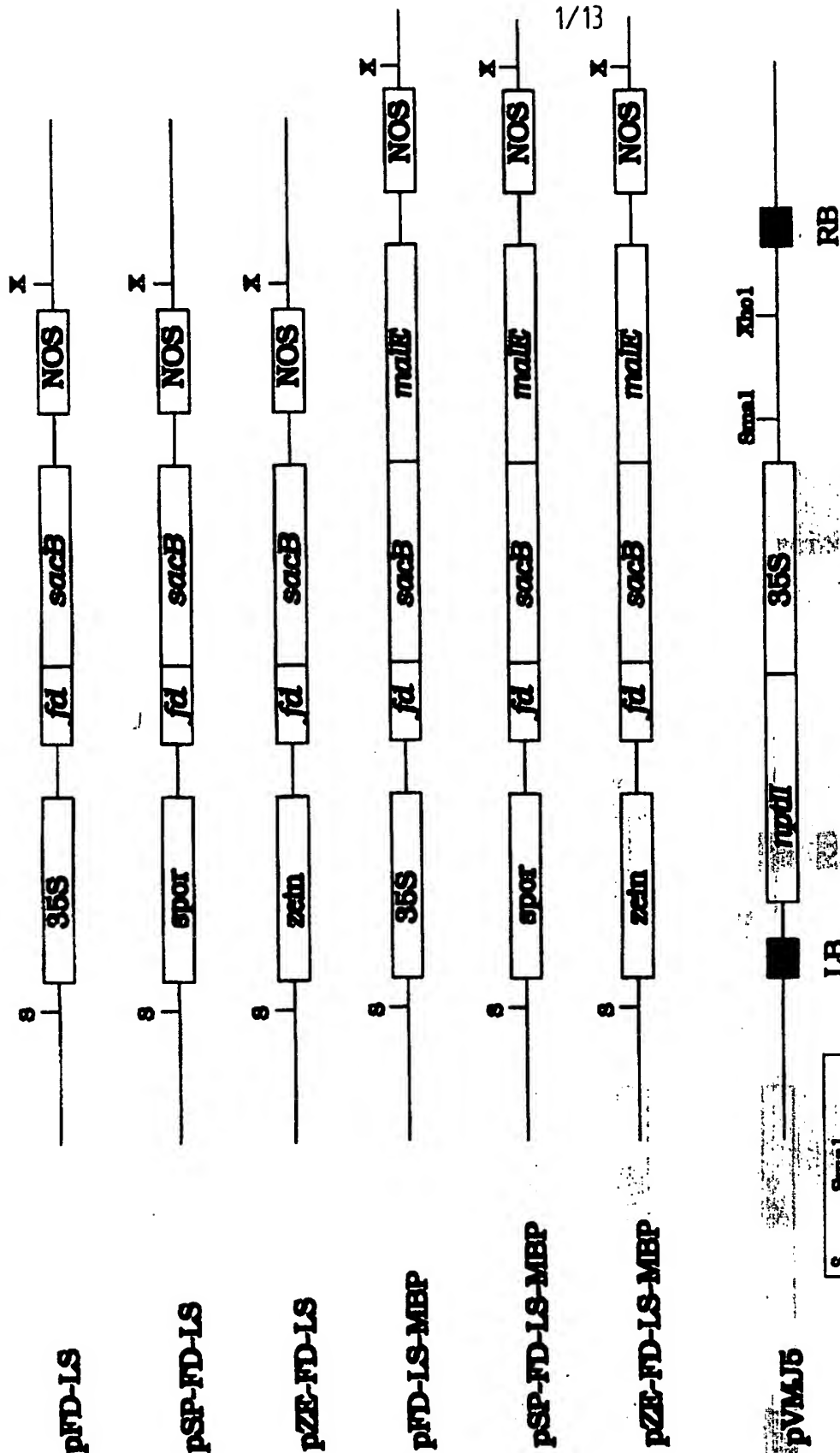
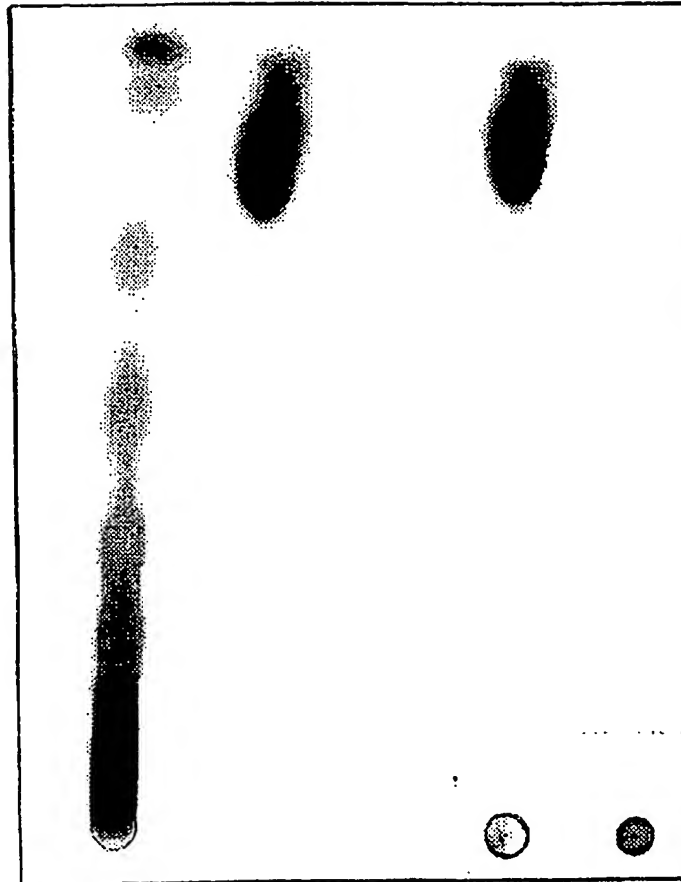


FIG. 1

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- F
- S

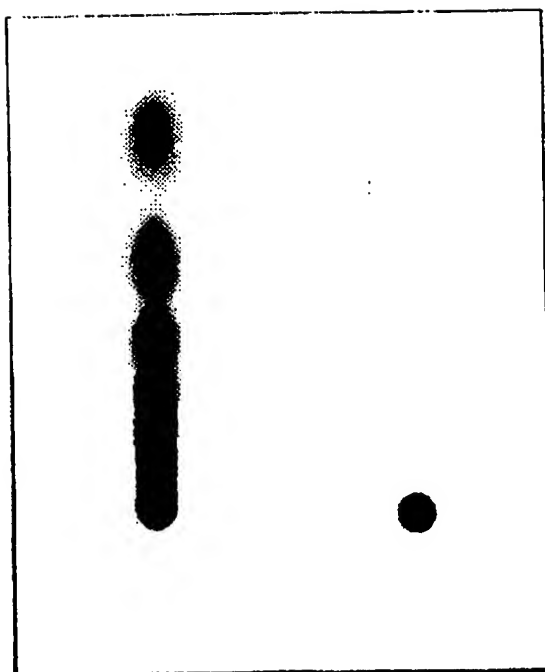


| | | | | |
|---|-------|---|-------|---|
| H | T | C | T | C |
| | <hr/> | | <hr/> | |
| | WT | | fd-LS | |

FIG. 2

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M WT fd-LS

FIG. 3

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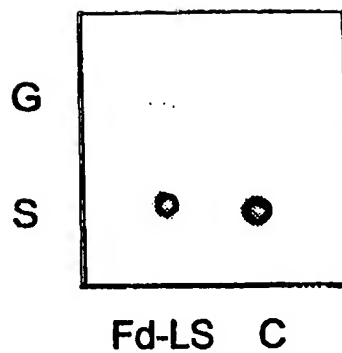
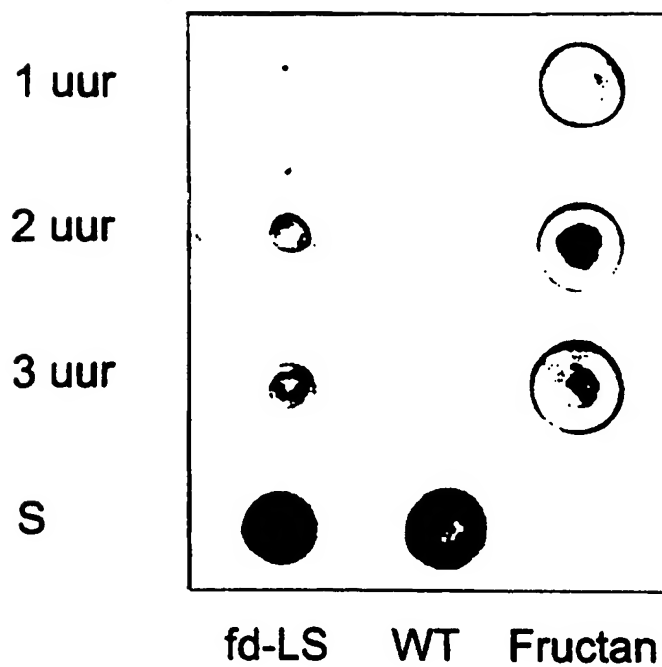


FIG. 4

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FIG. 5

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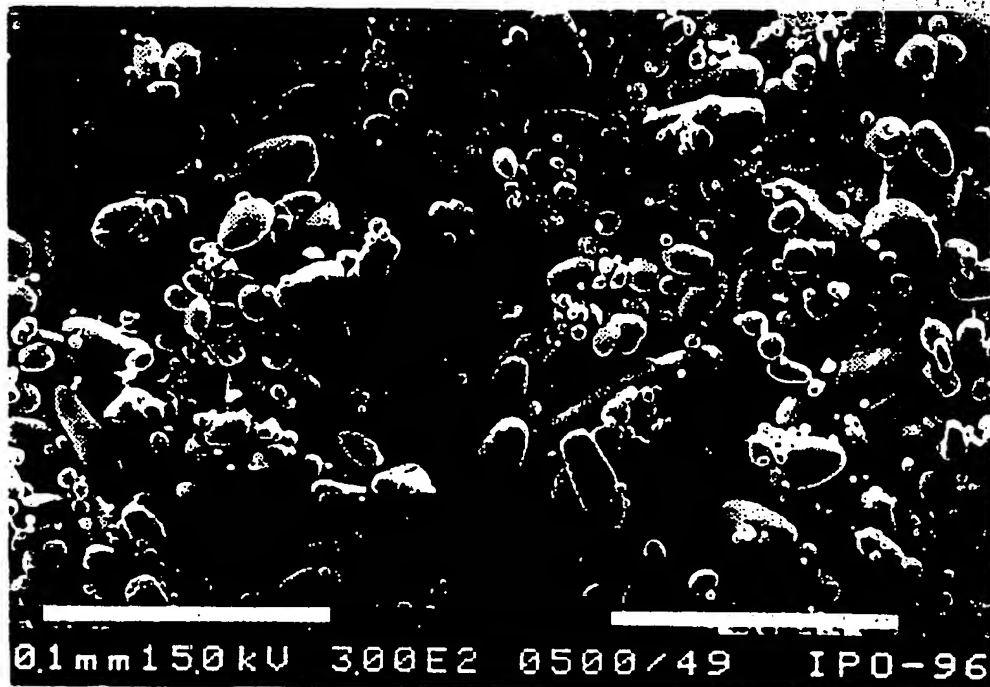
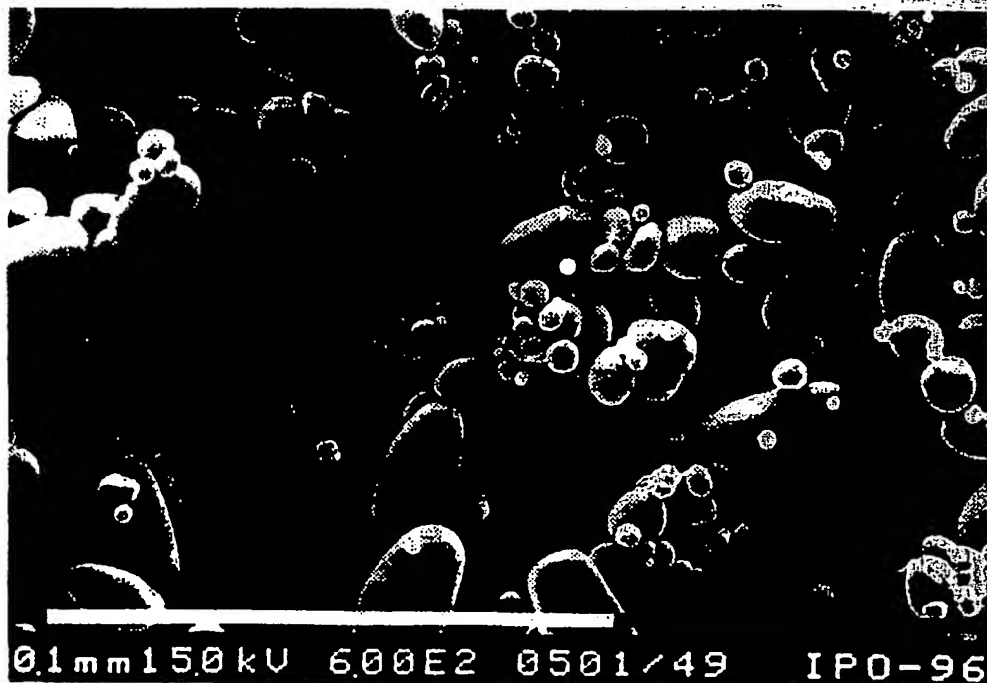
FIG. 6a "Wild type"



FIG. 6b "Wild type"

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FIG. 6c "192-3"FIG. 6d "192-3"

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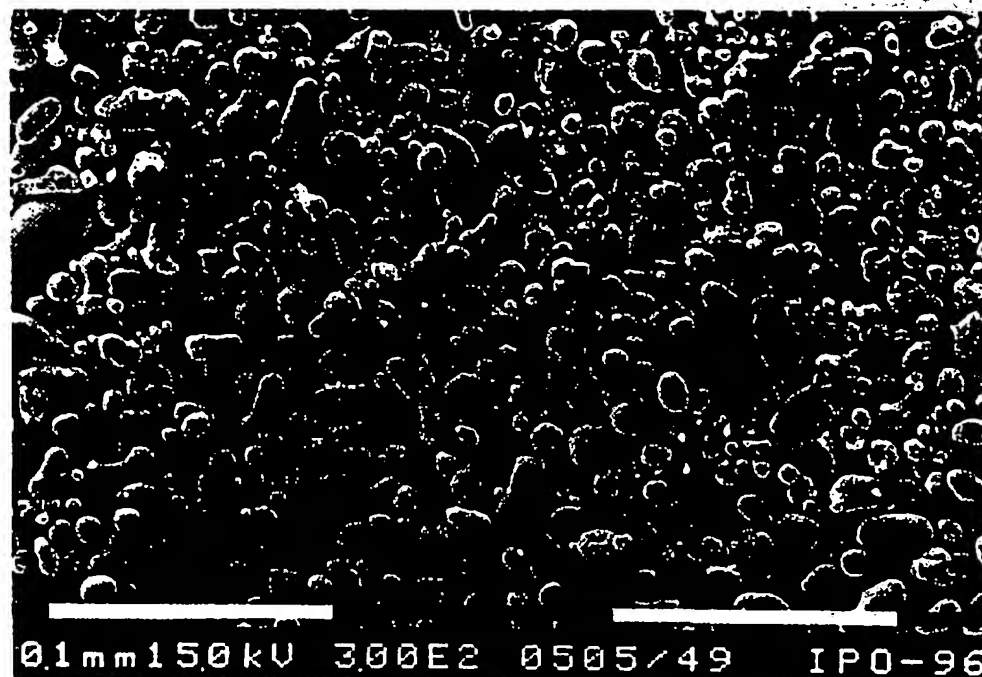


FIG. 6e "192-2"



FIG. 6f "192-2"

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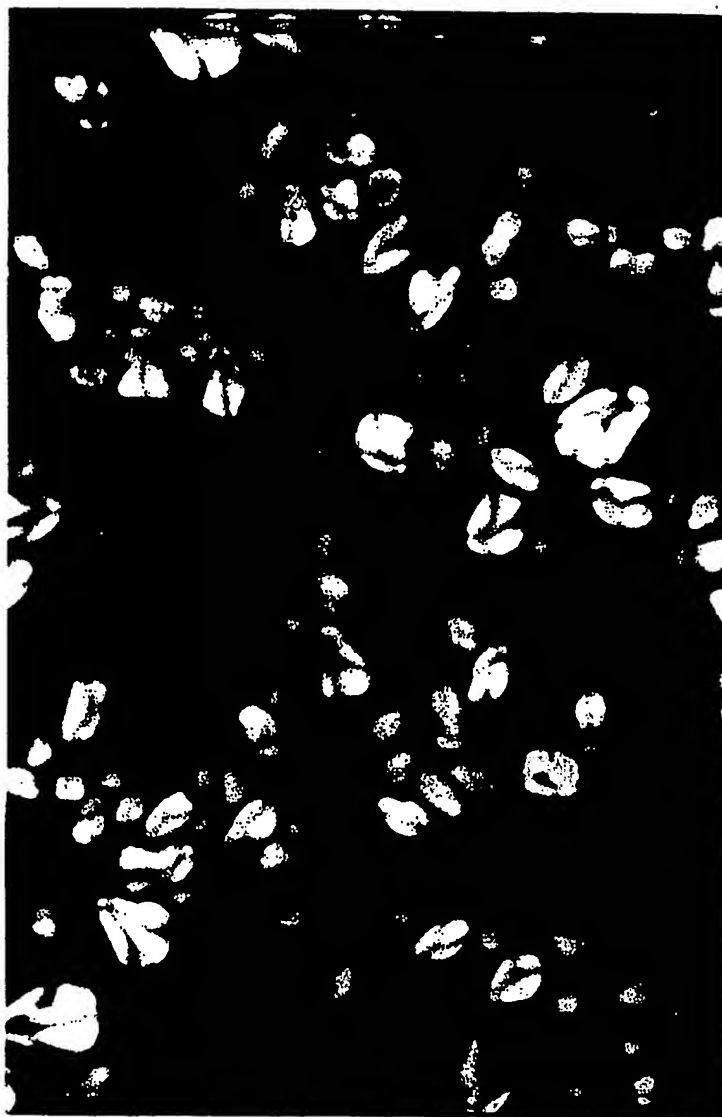


FIG. 7a "Wild type"

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FIG. 7b "192-3"

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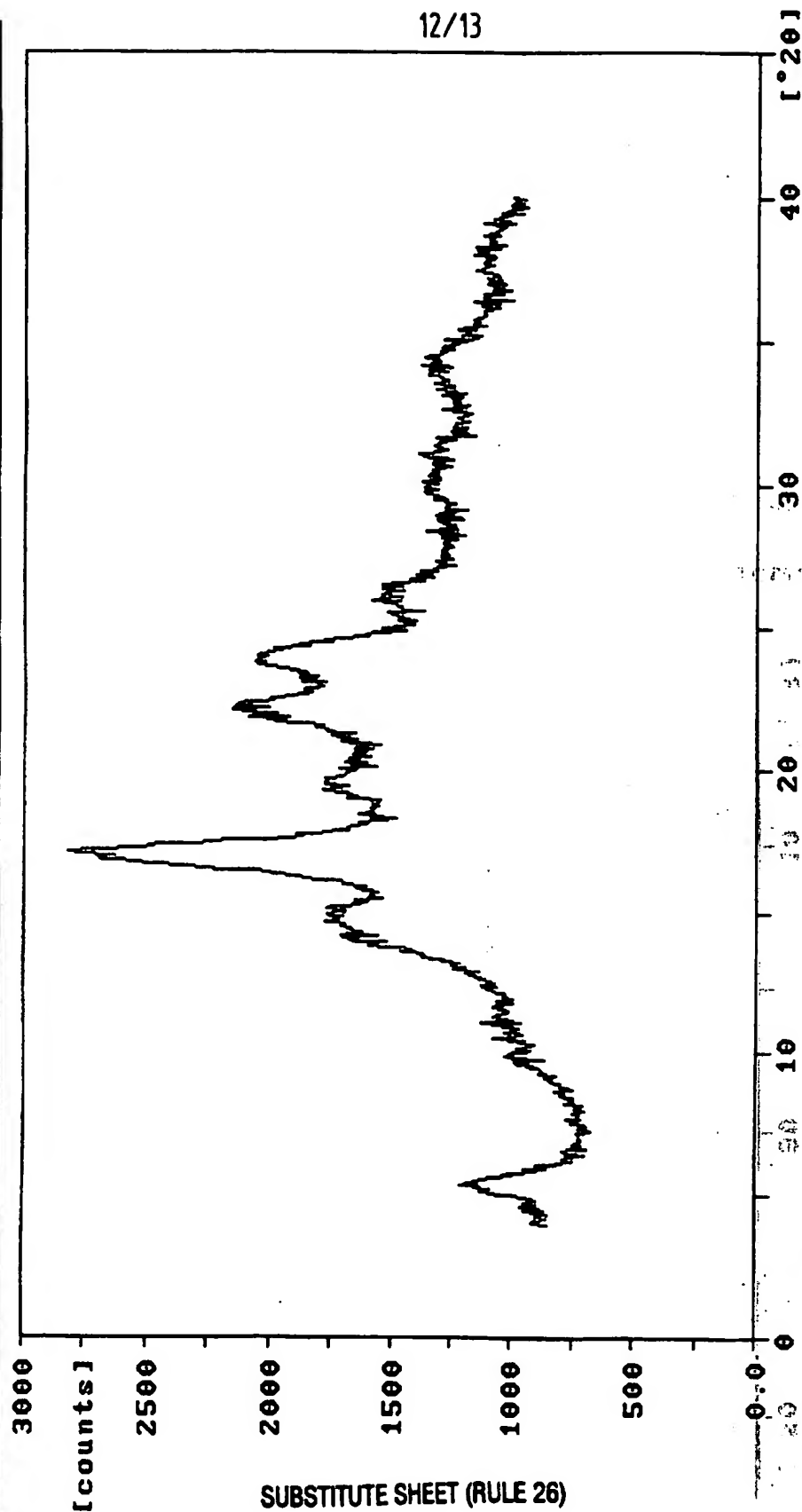


FIG. 7c "192-2"

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8-Nov-1996 21:20

Sample Identification: van der Have UT



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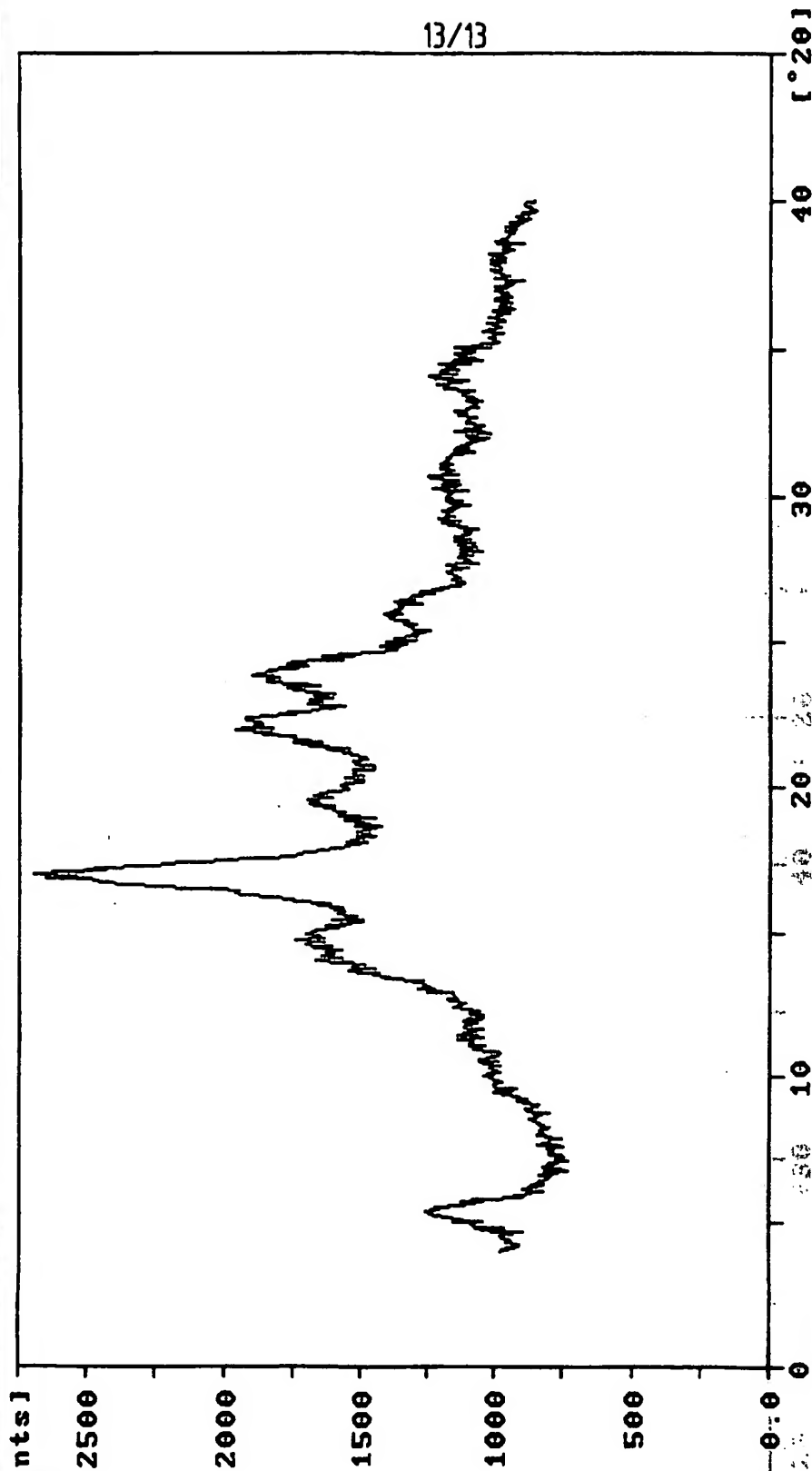
HAVEUT.RD

FIG. 8a

FIG. 8b

8-Nov-1996 20:23

Sample Identification: van der Have 192-3



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HAVE1923.RD

FIG. 8b

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00039

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/10 C12N15/82 C12N15/54 C12P19/04 A01H5/00
A23L1/00 A23L1/052

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|---|
| X | WO 94 14970 A (STICHTING SCHEIKUNDIG ONDERZOE ;SMEEKENS JOSEPHUS CHRISTIANUS (NL)) 7 July 1994 see the whole document --- | 1-4, 10, 12, 15, 17, 21-24, 33-35 |
| X | DE 42 27 061 A (INST GENBIOLOGISCHE FORSCHUNG) 17 February 1994 see the whole document --- | 1-4, 15, 17, 21, 22, 24, 33-35 |
| X | DE 44 20 223 C (INST GENBIOLOGISCHE FORSCHUNG) 4 May 1995 see the whole document --- | 1, 21, 22, 24 |
| | -/-- | |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

16 April 1997

Date of mailing of the international search report

24.06.1997

Name and mailing address of the ISA

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Holtorf, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00039

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|---|
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| X | WO 89 12386 A (CALGENE INC) 28 December 1989 see the whole document --- | 1-4,10, 15-18, 21-23, 33-35 |
| X | WO 96 01904 A (STICHTING SCHEIKUNDIG ONDERZOE ;SMEEKENS JOSEPHUS CHRISTIANUS (NL)) 25 January 1996 see the whole document --- | 1-4,10, 13,15, 17, 21-23, 33-35 |
| X | THE PLANT CELL, vol. 6, April 1994, pages 561-570, XP002020552 VAN DER MEER, I.M., ET AL.: "FRUCTAN AS A NEW CARBOHYDRATE SINK IN TRANSGENIC POTATO PLANTS" see the whole document --- | 1-4,10, 15,17, 21,22, 33-35 |
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INTERNATIONAL SEARCH REPORT

Intern. application No.

PCT/NL 97/ 00039

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

18 inventions * see continuation-sheets PCT/ISA/210 *

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-13,15,17,19-35 partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1 - Claims 1-13,15,17,19-35 partially

Method for the *in vivo* production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme levansucrase. Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

2 - Claims 1-13,15,17,19-35 partially

Method for the *in vivo* production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme sucrose-sucrose-fructosyltransferase (SST). Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

3 - Claims 1-13,15,17,19-35 partially

Method for the *in vivo* production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme fructan-fructan-fructosyltransferase (FFT). Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

4 - Claims 1-13,15,17,19-35 partially

Method for the *in vivo* production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme sucrose-fructan-fructosyltransferase (SFT). Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

5 - Claims 1-13,15,17,19-35 partially

Method for the *in vivo* production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucose-fructan-fructosyltransferase (GFT). Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

6 - Claims 1-13,16,18,19-35 partially

Method for the *in vivo* production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme altamansucrase. Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

7 - Claims 1-13,16,18,19-35 partially

Method for the in vivo production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucosyltransferase-I (GTF-I). Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch.

8 - Claims 1-13,16,18,19-35 partially

Method for the in vivo production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucosyltransferase-S (GTF-S). Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch.

9 - Claims 1-13,16,18,19-35 partially

Method for the in vivo production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucosyltransferase-SI (GTF-SI). Furthermore, the method to influence the size of starch-granules by applying special growth-conditions and use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch.

10 - Claims 1,14,15,17,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme levansucrase. Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch.

11 - Claims 1,14,15,17,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme sucrose-sucrose-fructosyltransferase (SST). Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

12 - Claims 1,14,15,17,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme fructan-fructan-fructosyltransferase (FFT). Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

13 - Claims 1,14,15,17,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme sucrose-fructan-fructosyltransferase (SFT). Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

14 - Claims 1,14,15,17,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucose-fructan-fructosyltransferase (GFT). Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

15 - Claims 1,14,16,18,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme alternansucrase. Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

16 - Claims 1,14,16,18,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucosyltransferase-I (GFT-I). Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

17 - Claims 1,14,16,18,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucosyltransferase-S (GFT-S). Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

18 - Claims 1,14,16,18,21-28 partially

Method for the in vitro production of modified polysaccharides through expression of a sugar-group-transferring enzyme, limited to the enzyme glucosyltransferase-SI (GFT-SI). Furthermore, the use of the modified polysaccharides for food and non-food applications, especially altering the retrogradation of starch .

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/NL 97/00039

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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